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# *HSP70* Gene Expression Pattern in Peripheral Blood Mononuclear Cells during Different Seasons in Pandharpuri Buffaloes

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## ABSTRACT

**Background:** Heat shock protein is one of the most abundant and best characterized heat shock protein family that consists of highly conserved stress proteins. Amongst these heat shock protein family, *HSP70* is expressed in response to stress and plays crucial roles in environmental stress tolerance and adaptation. Thermal stress alters the normal body homeostasis and causes severe detrimental effects on production and productivity of animals and impairs growth, immune status of animal. Therefore, the present study is proposed to study *HSP70* gene expression pattern in peripheral blood mononuclear cells.

**Methods:** The present study was conducted to study the relative mRNA expression pattern of *HSP70* gene in peripheral blood mononuclear cells (*PBMCs*) during different seasons in Padharpuri buffaloes. 10 Apparently healthy, non - pregnant Pandharpuri buffaloes (above 2 years of age) were taken for study. Blood samples were collected thrice i.e. once in winter season, once in summer season and once in rainy season. Real-time polymerase chain reaction was applied to investigate *mRNA* expression of *HSP70* gene during different seasons. Specificity of the desired products was documented using analysis of the melting temperature and high resolution gel electrophoresis to verify that the transcripts are of the exact molecular size predicted.

**Result:** To investigate the variation in relative mRNA expression profile of *HSP70* gene, Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH* gene) was used as a housekeeping gene. The relative expression values of *HSP70* during summer and rainy seasons were found statistically significant in comparison to winter. These results suggest that *HSP70* gene expression varies with season and this variation may play an imperative role in conferring thermo tolerance against heat stress during different seasons.


**Key words:** HSP70 Gene expression, Pandharpuri buffalo, PBMC, Seasons.

## INTRODUCTION

Livestock plays an important role in rural economy through its contribution to food, employment generation and drought power. Buffalo has been the mainstay of rural economy in the Indian subcontinent and South East Asian countries. The world buffalo population is 204 million in forty two countries of which 97% population is confined to Asia and India (FAO, 2021). India ranks first in buffalo population in Asia and has 113.33 million buffaloes (DAHD, 2019). Buffalo is predominantly distributed in different regions of the country and well adopted to different agro climatic conditions due to its ability to sustain and thrive on poor quality roughages and better ability to resist tropical diseases. These buffaloes are concentrated in Pandharpur, North Solapur, South Solapur, Akkalkot, Sangola and Mangalvedha tehsils of Solapur district; Miraj, Walwa, Tasgaon tehsils of Sangli district of Maharashtra state (NDDB, 2015). The region of Satara district is heterogeneous in its *agro*-ecology due to diversities in its physiographic and *climatic* profile and is located between 17°5' and 18°11' north latitudes and 78° 33' and 74° 54' east longitudes. Although this buffalo breed called "Pride of Western Maharashtra", but full potential of this animal however still remained to be exploited for rural upliftment. Heat stress has various detrimental effects on livestock (Marai and Habeeb, 2010). Further, humidity becomes more important since it directly affects the evaporation rate. Therefore, the temperature humidity index (*THI*) becomes relevant under conditions of high temperature and high humidity. Temperature-humidity index (*THI*), a parameter that is extensively used to describe heat load on animal and humans, is a good indicator of stressful thermal climatic conditions (McDowell *et al*., 1976).

*HSP70* is known to be a highly inducible chaperon and plays a key role to stabilize the native conformation of proteins and maintenance of cell survivability during thermal stress (Beckham *et al*., 2004). Particularly in mammals,

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exposure to hypothermia or hyperthermia has been related to morphological and physiological modifications. Heat shock proteins (*HSP*s) are multigene families that range in molecular size from 10-150 kDa and are found in all major cellular compartments (Patir and Upadhyay, 2010). They are highly conserved proteins present in all the cells of living organisms and are essential for cellular viability as these have major physiological roles in protein homeostasis. Dangi *et al*., (2012) revealed that 70-kDa heat shock protein family assists the folding of proteins upon translation in the cytosol of both prokaryotic and eukaryotic cells as determined by genetic and biochemical analyses.

Buffaloes are seasonally polyestrous with a marked reduction in their reproductive performance during summer in regions of high latitude. Relative expression of *HSP70* genes varied markedly among the heat- and cold-adapted goat breeds with a moderate variation between breeds and showed a good response to increased or decreased ambient temperature (Banerjee *et al*., 2014, in caprine *PBMC*s (Gupta *et al*., 2013), leukocytes of buffalo (Pawar *et al*., 2014), Murrah buffalo (Mishra *et al*., (2010), dermal fibroblasts of cattle (Singh *et al*., 2014). In addition, it is speculated that heat shock stress effects on the early embryo directly and it also influences the development to the late embryo (Wee *et al*., 2008). Heat stress in farm animals, such as cattle and buffalo during summer and post-summer seasons is a problem for livestock producers. The effect of heat stress becomes pronounced when heat stress is accompanied with ambient humidity impairing the immune status, growth, production and reproductive performance of animals (Mishra *et al*., 2010). No studies have been designed so far to gain an insight into the seasonal variation effects on *HSP70* gene expression profile in Pandharpuri buffalo. The present study is therefore, proposed to study *HSP70* gene expression pattern in peripheral blood mononuclear cells.

## MATERIALS AND METHODS

### Sample collection

The experiment was conducted on 10 apparently healthy Pandharpuri buffaloes above 2 years of age maintained under loose housing condition. The meteorological variables like temperature (dry bulb and wet bulb) and relative humidity were recorded for the Month of May (summer season), for the month of August (rainy season) and for the month of December (winter season) and were used for calculation of temperature humidity index (*THI*) as per the formula given by Madar *et al*. (2006). 5 ml blood samples from these animals were collected aseptically by jugular vein puncture during peak winter season, peak summer season and peak rainy season. Safety measures were taken to minimize the effect of ribonuclease during processing. All samples were processed within one hour of collection.

### *PBMC*s isolation

Dilution of whole blood was done in phosphate buffer saline (PBS, pH=7.4) in the ratio of 1:1. *PBMC*s were isolated by using HiSepTM Lymphocyte separation media 1077 (*LSM*) (Himedia). *LSM* was aseptically transferred to a 15 ml clean centrifuge tube and overlaid with diluted blood to produce a clean interface between the two layers. The mixture was centrifuged at 1500 rpm for 30 min. at room temperature. *PBMC*s fraction from the interface was collected gently. Further centrifugation was done for washing the cells with phosphate buffer saline (*PBS*) (pH 7.4). Red blood cells lysis buffer was added to *PBMC*s pallet, mixed well and centrifuged. Supernatant was discarded and washing was repeated twice. Finally, *PBMC*s pellet was re-suspended and transferred to a sterile DEPC micro-centrifuge tube.

### Total *RNA* extraction and quality determination

Total *RNA* was isolated using Trizol reagent (SRL). Trizol reagent and chloroform were added to *PBMC*s pellet and mixed gently followed by centrifugation at 12,000 rpm for 15 min at 4°C. The initial upper aqueous layer was aliquoted into sterile microcentrifuge tubes and equal volume of ice cold isopropanol was added, vortexed gently followed by centrifugation at 12,000 rpm for 12 min. 4°C. Pellet was washed twice with 75% ethanol by centrifugation at 7500 rpm for 5 min at 4°C. The isolated total *RNA* was stored in nuclease free water at 4°C (Qiagen, India). Purity of *RNA* was checked by using NanoDrop spectrophotometer (ARGLabs). 2 µl of dissolved *RNA* was added to find out the ratios of O.D. at 260 nm and 280 nm. Quality of *RNA* was assessed by electrophoresis on a denaturing agarose (1.5% w/v gel). 30 ml of 1.5% agarose gel was used along with 4 µl Ethidium bromide for staining of the bands. The *RNA* suspension was further processed for *cDNA* preparation.

### Reverse Transcription and Quantitative Real-Time PCR

1 µg of total *RNA* were reversed transcribed to complementary *DNA* (*cDNA*) using *cDNA* synthesis kit (Fermentas) according to manufacture instructions. First strand *cDNA* was confirmed by amplification of *GAPDH* gene.

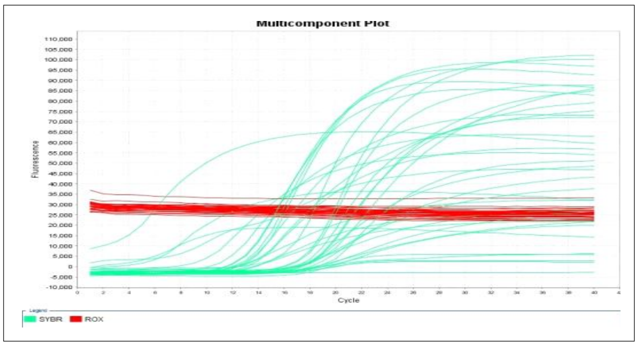
### Primers

Primers were designed for *HSP70* by the Integrated DNA Technologies (IDT) using Beacon software. The sequences and expected polymerase chain reaction (*PCR*) product lengths are shown in Table 1. Quantitative Real-time *PCR* (*qPCR*) was performed with Invitrogen Sybr green ®Supermix kit. The *qPCR* conditions were as follows, initial denaturation at 95°C for 30 s, annealing at 58°C for 10 s and lastly extension at 72°C for 15 s for 35 cycles. No template control (*NTC*) was placed for gene quantification for checking the contamination in the reaction components other than the *cDNA*. After the run has ended, cycle threshold (Ct) values and amplification plot for all determined factors were acquired by using the “dissociation curve” method of the real time machine (Applied Biosystem, USA). The specificity of real time *PCR* products were checked by analysis of melting temperature (Tm) of the product obtained from dissociation or melting curve and by 1.5% agarose gel electrophoresis to verify the exact amplicon size. Relative expression of *PCR* product was determined by the equation suggested by Pfaffl (2001). The relative expression of *HSP70* gene with *GAPDH* as reference gene was determined in Pandharpuri buffalo using Pfaffl method (2001) and with using Completely Randomized Design cited by Snedecor and Cochran (1980). The significance of analysis was determined at probability levels of 95 per cent ($P<0.05$).

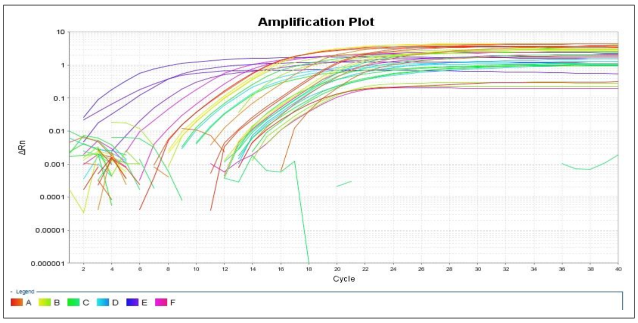
## Results and Discussion

### Temperature-humidity index

Mean values of temperature humidity index (*THI*) during different seasons of the study period are presented in the Table 2. During the study period, Mean values of *THI* in summer, rainy and winter season was found to be 76.97± 0.38, 73.32± 0.38 and 68.81± 0.49. The highest *THI* was recorded during the peak summer season and lowest *THI* was recorded during the peak winter season. The *THI* recorded was significantly ($P<0.05$) higher in peak summer season as compared to the rainy and winter season. *THI* of summer, rainy and winter seasons differed significantly ($P<0.05$). Our results are consistent with the observation made by Baumgard *et al*. (2006) in dairy cattles, who reported that *THI* > 72 was the point at which a dairy cow starts to decrease productivity as in high temperature. In this experiment, *THI* of 72 can be achieved at moderate temperatures if relative humidity is high. Further, effect of heat on dairy cattle maintenance and milk production is heavily influenced by relative humidity.




**Picture 1:** Multicomponant plot of gene expression.




**Picture 2:** Amplification plot for *HSP70* gene expression.

### Relative expression profile of *HSP70* gene

The expression of *HSP70* gene showed temperature sensitivity and seasonal variation. Relative expression of *HSP70* gene varied markedly among different seasons (Fig 1). Statistical analysis revealed a significant variation between different seasons ($P<0.01$) for all *HSP70* gene expression. The expression of *HSP70* gene was significantly ( P Ã 0.05) higher in summer season as compared to the rainy and winter season and the relative *mRNA* expression of *HSP70* was very low in winter season. Amplification plots and multicomponent plot for all the reactions of *HSP70* gene expression was analyzed to check unspecific binding, primer dimer formation or secondary structure formation. Single peak in all experiments during *qPCR* signified that the primers were highly specific to the target and there was not any primer dimmers formation (Picture 1) and amplification plot for *HSP70* gene and *GAPDH* expression depicted in Picture 2 and confirmation of *qPCR* products on 1.5% agarose gel electrophoresis showed in Picture 3. The expression studies indicated that of *HSP70* gene was up regulated during both summer and rainy seasons whereas *HSP70* gene was observed to be down regulated during winter season. An increased *mRNA* expression in

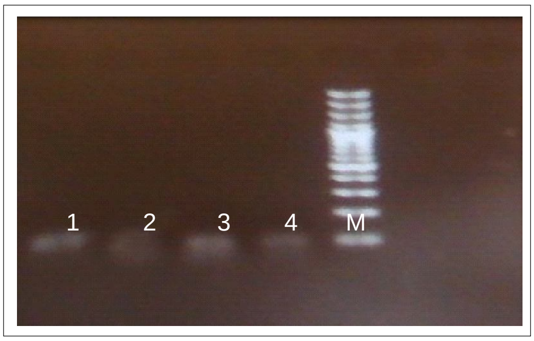
**Table 1:** Primers sequences, annealing temperature (TA) and size of amplicons for each specific gene used in the gene expression evaluation.

| Gene | Primer sequence | TA(0) | Amplicon size (bp) |
|---|---|---|---|
| HSP70 | F-5′GGACAAGGCGCAGATCCA 3′ | | |
| | R-5′AAGAAGTCCTGCAGCAGCTT 3′ | 63 | 84 |
| GAPDH | F-5′CTGCAACCCAGAAGAC TGT 3′ | | |
| | R-5′GCCAGTAGAAGCAGGGATGATATTC 3′ | 54 | 107 |

**Table 2:** Mean±S.E. values of temperature humidity index (THI) during different seasons.

| Month/seasons | Tdb (°C) | Twb (°C) | Relative humidity (%) | Temperature humidity index (THI) | C.D. values |
|---|---|---|---|---|---|
| May/summer | 27.87±0.26 | 21.94±0.28 | 59.74±0.77 | 76.97$^{a}$±0.38 | (0.01) = 1.65* |
| August/rainy | 23.46±0.23 | 22.35±0.08 | 87.55±1.10 | 73.32$^{b}$±0.38 | (0.05) = 1.24** |
| December/winter | 20.88±0.30 | 14.48±0.42 | 82.19±0.78 | 68.81$^{c}$±0.49 | |

Mean THI value with different superscripts differ significantly at 1% and 5% level of significance




**Picture 3:** Confirmation of real time PCR products on 1.6% agarose gel electrophoresis.
Lane M: 100 bp DNA ladder, Lane 1-4: *HSP70* gene (132 bp).

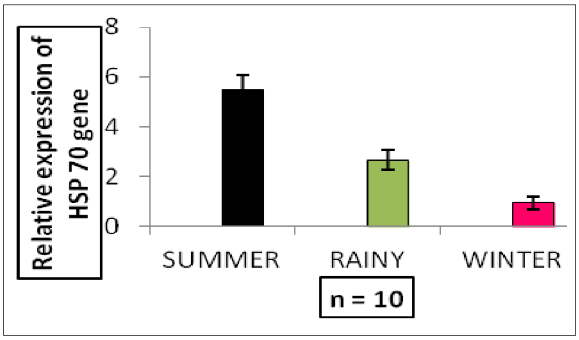



**Fig 1:** Relative expression pattern of *HSP 70* gene during different seasons.

Pandharpuri buffalo during summer season was found to be more than 2 fold and 5 fold than in rainy and winter seasons respectively (Fig 1). During summer season, *HSP70* gene expression was found statistically significant (P>0.05) with compared to rainy season.

In our study, it has been observed that the expression of *HSP70* was significantly higher during the summer season as compared to the winter season in Pandharpuri buffalo, which might play an important role in thermal stress tolerance against harsh environmental conditions. Thermal stress induces differential gene expression and biochemical response at the cellular level. Our findings corroborates with the reports of Dangi *et al*., (2012) who reported that individuals exposed to stress elicit *HSP* response in the cells of various organs and higher expression of *HSP70* at the tissue level provides protection to cells during chronic heat stress. Investigations in the present study are corroborated by Parmar *et al*., (2015) who reported *HSP70* gene expression pattern in Sahiwal cows during different. Our research findings corroborate with the previous studies, where thermal stress induced rise in *HSP70* expression in caprine *PBMC*s (Gupta *et al*., 2013), leukocytes of buffalo (Pawar *et al*., 2014), in thigh muscle and colon tissue of Ghungroo and Large White Yorkshire (Parkunan *et al*., 2015), Murrah buffalo (Mishra *et al*., (2010), dermal fibroblasts of cattle (Singh *et al*., 2014), in bovine ovary (Velazquez *et al*., 2011) and bull sperms (Rajoriya *et al*., 2014). Heat stress reduces the efficiency of animal production leading to multibillion dollar losses to global animal agriculture (Bernabucci *et al*., 2010). Further, Collier *et al*., (2008) reported that cellular responses to heat stress include activation of heat shock transcription factor 1. In the present study, increased expression pattern of *HSP70* gene in summer season than that of rainy and winter seasons in Pandharpuri buffalo may be due to the fact that *HSPs* provide signaling to the immune system to encourage increased killing of pathogenic bacteria by neutrophils and macrophages and other innate immune cells against invading bacteria. The result obtained in this experiment confirms the results of earlier studies by Kapila *et al*., (2013) on *HSP70* gene expression pattern in buffalo and suggested that heat stress condition in summer showed immediate induction in their expression after heat shock and remained up regulated after exposure to 42°C for one hour.

The findings of present study suggested that expression of *HSP70* is influenced by the *THI* of the season and its up-regulation during high *THI* may play a crucial role in providing defence against thermal injury at cellular level. In our study, the expression of *HSP70* gene in Pandharpuri buffalo was dependent on heat stress during summer season at 41.10 ±0.32°C and 86.00±2.06 % RH under present finding. This heat stress may be due to high environmental temperature influence on *HSP70* gene expression in these buffaloes. Pandharpuri buffalo being an indigenous breed of western Maharashtra, it is well developed defence mechanism involving the maintenance of high constitutive level of *HSP70* gene in their *PBMC* as a mechanism for the protection against summer periods of extreme heat stress and hence, the present studies on Pandharpuri buffalo indicated that *HSP70* gene induction takes place due to heat exposure during summer season.

## CONCLUSION

The expression of *HSP70* gene in Pandharpuri buffalo during summer seasons was found to be more than 2 fold and 5 fold than that of the rainy and winter seasons respectively. Hence, it is considered that *HSP* is most sensitive and reliable marker of heat stress in this breed. Further, it has been suggested that *HSP70* gene influenced by *THI* values and shows possible involvement to ameliorate deleterious effect of thermal stress so as to maintain cellular integrity and homeostasis. *HSP70* gene expression in these breed has significant implication in development of strategies to cope with challenges with climate change.

**Conflict of interest:** None.

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